dMyc is required for larval growth and endoreplication in *Drosophila*

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Accepted 3 February 2004

Development 131, 2317-2327
Published by The Company of Biologists 2004
doi:10.1242/dev.01108

Summary

Members of the Myc family of proto-oncogenes have long been implicated in regulating proliferation, apoptosis and oncogenesis. Recently, transcriptional and biological studies have suggested a direct role for Myc in regulating growth. We have used *dm^4*, a new null allele of the *Drosophila diminutive* (*dm*) gene, which encodes dMyc on the X chromosome, to investigate a role for dMyc in larval endoreplicating tissues, where cellular growth and DNA replication occur in the absence of cell division. Hemizygous *dm^4*/Y mutants arrest as second instar larvae, and fat body nuclei of *dm^4*/Y mutants fail to attain normal size and normal levels of DNA, resulting from a reduced frequency of S-phase. Thus, dMyc is required for endoreplication and larval growth. In support of this, dMyc, as well as its antagonist dMnt, are expressed in larval tissues in a pattern consistent with their involvement in regulating endoreplication. Overexpression of dMyc in endoreplicating cells results in dramatic increases in nuclear DNA content and cell and nucleolar size, whereas dMnt overexpression has the opposite effect. BrdU incorporation and Cyclin E protein levels continue to oscillate in dMyc-overexpressing cells, indicating that the normal cell cycle control mechanisms are not disrupted. dMyc driven growth and endoreplication are strongly attenuated when the endocycle is blocked with Cyclin E or the cdk inhibitor p21. By contrast, the ability of dMyc to promote growth and endoreplication is only partly reduced when PI3K activity is blocked, suggesting that they influence distinct growth pathways. Our results indicate that larval growth and endoreplication are coupled processes that, although linked to cell cycle control mechanisms, are regulated by dMyc and dMnt.

Key words: Myc, dMyc, *diminutive*, Endoreplication, Polyploid, Growth, Cyclin E, Mnt, dMnt, Fat body, Salivary gland, p21, *Drosophila*

Introduction

The Myc/Max/Mad network of transcription factors plays a role in a wide range of cellular processes including growth and proliferation, differentiation, apoptosis and oncogenesis (reviewed by Grandori et al., 2000; Nesbit et al., 1999; Oster et al., 2002; Schmidt, 1999; Zhou and Hurlin, 2001). Members of the Myc family are found overexpressed in a wide variety of human tumors and are implicated in tumor initiation and progression. When ectopically expressed in mammalian cells, Myc proteins can induce proliferation and growth, block terminal differentiation and, in cooperation with other signals, induce transformation (reviewed by Grandori et al., 2000; Oster et al., 2002). Conversely, overexpression of Mad family members leads to arrest of the cell cycle in G1 (reviewed by Zhou and Hurlin, 2001).

Myc and Mad proteins are thought to regulate these processes through transcriptional control of genes required for growth and proliferation. Both Myc and Mad form heterodimeric complexes with their common binding partner Max, mediated by the basic helix-loop-helix zipper (bHLHZ) domains present in all these proteins. Myc-Max and Mad-Max heterodimers bind DNA by recognizing the E-box sequence CACGTG. Myc-Max heterodimers recruit the co-activators TRRAP and p300, resulting in transcriptional activation of genes containing promoter-proximal E-box sequences, most probably through acetylation of histones in chromatin. By contrast, Mad-Max heterodimers recruit the Sin3 co-repressor, which binds histone deacetylases to deacetylate histones and thereby antagonize Myc/Max activity (reviewed by Grandori et al., 2000; Oster et al., 2002). In addition, Myc, by inactivating the Miz-1 transcription factor, represses some genes (such as a subset of cyclin dependent kinase inhibitors) that negatively regulate growth and proliferation (Seoane et al., 2002; Staller et al., 2001).

The Myc/Max/Mad network is conserved in *Drosophila* and comprises dMax and single members of the Myc and Mad families, dMyc and dMnt, respectively (Gallant et al., 1996; Schreiber-Agus et al., 1997) (L. Loo, J. Secombe and R.N.E., unpublished). Both loss-of-function and overexpression studies have demonstrated an important role for dMyc, which is encoded by the *diminutive* (*dm*) gene, in regulating cellular growth. Hypomorphic *dm* mutants are female sterile and all mutant adults are smaller than wild type with thinner, shorter bristles (Gallant et al., 1996; Johnston et al., 1999; Schreiber-Agus et al., 1997). The reduced body size of these mutants results from a decrease in cell size and possibly cell number

(Johnston et al., 1999). Conversely, ectopic expression of dMyc in wing disc cell clones increases cell size and promotes G1/S progression without altering cell doubling time.

The notion that dMyc is involved in growth is consistent with more recent studies attempting to define Myc target genes in vertebrates and flies. Genomic analyses to identify expression changes induced by Myc and Mad (Boon et al., 2001; Coller et al., 2000; Guo et al., 2000; Iritani and Eisenman, 1999; Neiman et al., 2001; Watson et al., 2002), as well as direct DNA binding sites for mammalian Myc (Fernandez et al., 2003; Li et al., 2003) and *Drosophila* dMyc, dMax and dMnt (Orian et al., 2003) have, in general, shown that Myc regulates a large and diverse set of genes whose functions are consistent with the ability of Myc to promote growth and proliferation. The *Drosophila* larva consists predominantly of differentiated polyploid tissues that support the growth and eventual metamorphosis of the mitotic imaginal discs and nervous system, which will give rise to the tissues of the adult animal (see Gilbert, 2003). Although growth of the imaginal discs is accomplished by cell growth coupled to proliferation, growth of the polyploid tissues occurs in conjunction with endoreplication, a modified cell cycle, in which DNA replication results in polytene chromosomes. Endoreplicating cells dramatically increase in size without dividing (reviewed by Edgar and Orr-Weaver, 2001). Little is known about what regulates the endocycle in larval cells. However, it is clear both from the reproducible final tissue ploidies (Smith and Orr-Weaver, 1991) and the effect of nutrient deprivation on endoreplication (Britton and Edgar, 1998; Britton et al., 2002) that environmental factors and the regulation of developmental genes are important. Endocycles consist of distinct S and gap phases, regulated by oscillations in Cyclin E/cdk2 activity (reviewed by Edgar and Orr-Weaver, 2001). Oscillation of Cyclin E/cdk2 activity may be accomplished by the periodic expression of Dacapo, a $p27^{Cip/Kip}$ homolog that inhibits Cyclin E activity and oscillates out of phase with Cyclin E in endocycling ovarian nurse cells (de Nooij et al., 2000; Lilly and Spradling, 1996).

Because of the relationship of dMyc function to cell growth in mitotic cells, we have now investigated the roles of dMyc, as well as dMnt, in larval endoreplicating tissues and explored their potential functions in regulation of both growth and DNA endoreplication.

Materials and methods

Fly strains

Genotypes used in these experiments: *w*; iso2; 3, $P\{w^{+mGT}=GT1\}dm^{BG02383}$ w^{1118} and $P\{w^{+mGT}=GT1\}dm^{BG00605}$ w^{1118} (Lukacsovich et al., 2001), FM7i, Act-GFP, *w*; Act5C>CD2>Gal4, UAS GFP_{NLS} (Neufeld et al., 1998; Pignoni and Zipursky, 1997), *yw* $HS\text{-}FLP^{122}$, *w*; UAS-$dMyc^{42}$ (Zaffran et al., 1998), *w*; UAS-$dMnt^{35}$ (L. Loo, J. Secombe and R.N.E., unpublished), *w*; Adh-Gal4 (effector 1) (Fischer et al., 1988), ptc-Gal4, *w*; UAS-p60 (Weinkove et al., 1999), *w*; UAS-DmcycE (Neufeld et al., 1998), and *w*; UAS-p21 (Neufeld et al., 1998).

Deletion alleles of *dm* were generated by imprecise P-element excision, in flies carrying $P\{w^{+mGT}=GT1\}dm^{BG02383}$ alone or in trans to $P\{w^{+mGT}=GT1\}dm^{BG00605}$. Deletions in the *dm* gene were identified by PCR and confirmed by sequencing. To verify that the observed phenotypes were caused by the deletion in dm^4, dm^4/FM7i, Act-GFP females were crossed to males homozygous for a transgene in which the tubulin promoter drives dMyc expression (a kind gift from Peter Gallant). Progeny were scored for the presence of viable dm^4 males. dm^{1-14-2} retains all of the *dm* gene and 32 bp of $P\{w^{+mGT}=GT1\}dm^{BG02383}$ P-element sequence and is phenotypically wild type in all of our assays.

Western blotting

One hundred larvae per sample were homogenized and boiled in SDS sample buffer and run on 8% SDS-PAGE gels. Monoclonal antibodies were used to detect dMyc (P4C4-B10, undiluted) (Prober and Edgar, 2000) and actin (1:10,000, Sigma), which were visualized using HRP-conjugated secondary antibodies (Zymed) and SuperSignal ECL (Pierce).

Immunocytochemistry

Dissected larvae were fixed in 4% paraformaldehyde/PBS or 70% ethanol/PBS. Washes and antibody incubations were performed in 0.1% Tween-20 in PBS or 0.1% Triton and 0.1% BSA in PBS. Primary antibodies used were mouse anti-dMyc (P4C4B10, undiluted) (Prober and Edgar, 2000), mouse anti-dMnt (P5D6B8, undiluted) (L. Loo, J. Secombe and R.N.E., unpublished), mouse anti-BrdU (Becton-Dickinson, 1:100), mouse anti-Nop1p, for detection of fibrillarin, (1:600) (Aris and Blobel, 1988) and guinea pig anti-Cyclin E (1:1600) (T. Orr-Weaver). Alexa- (Molecular Probes) or FITC-conjugated (Zymed) secondary antibodies were used. DNA was visualized with 4′,6-Diamidine-2-phenylindole (DAPI) or propidium iodide. Larvae were fed 5-bromodeoxyuridine (BrdU) at 100 μg/ml.

Ectopic expression

Ectopic expression of UAS-regulated transgenes was driven by ADH or Ptc Gal4 drivers (Brand and Perrimon, 1993) or by using the Flp/Gal4 method (Neufeld et al., 1998; Pignoni and Zipursky, 1997), with or without a 1 hour 37°C heat shock during the first larval instar.

Quantification of DNA content

A Delta Vision microscope (Applied Precision) was used to take 0.2-0.5 μm sections through nuclei in the posterior salivary gland or fat body. The fluorescent intensity of single nuclei was calculated using the ImageQuant software package (Applied Precision).

RT-PCR

For RT-PCR analysis of *dm* mutants, RNA was isolated from larvae using TRIzol® Reagent (Invitrogen). PCR (94°C for 3 minutes; 25 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes; 72°C for 10 minutes) was performed to detect dMyc sequence, across the junction of exons two and three (primers: 5′-GAGCAACAACAGGCCATCGATATAG-3′, 5′-CCTTCAGACTG-GATCGTTTGCG-3′) and within exon three (5′-TGTGCAGAT-GAGGAAATCGATGTCG-3′, 5′-TGCGTCACTTTGTTATTGACT-CCC-3′), and CaMKII (5′-CAGTGGCGACTTTGATGGATACAC-3′, 5′-TGTAGCACTTTCATTAACATGTGC-3′). CaMKII was chosen as a control because overexpression of dMyc does not change its expression (Orian et al., 2003).

For RT-PCR from isolated tissues, RNA was isolated and reverse transcribed using the Cells-to-cDNA kit (Ambion). Forty-five cycles of PCR was performed to detect dMyc sequence, across the junction of exons two and three, and Rp49, using the above conditions.

Results

dMyc is required for larval growth

A hypomorphic allele of the *diminutive* (*dm*) gene, which encodes dMyc, is reported to exhibit a slight delay in development to the adult stage (Johnston et al., 1999), suggesting that reducing dMyc levels slows larval growth. To more effectively test the requirement for dMyc in larval

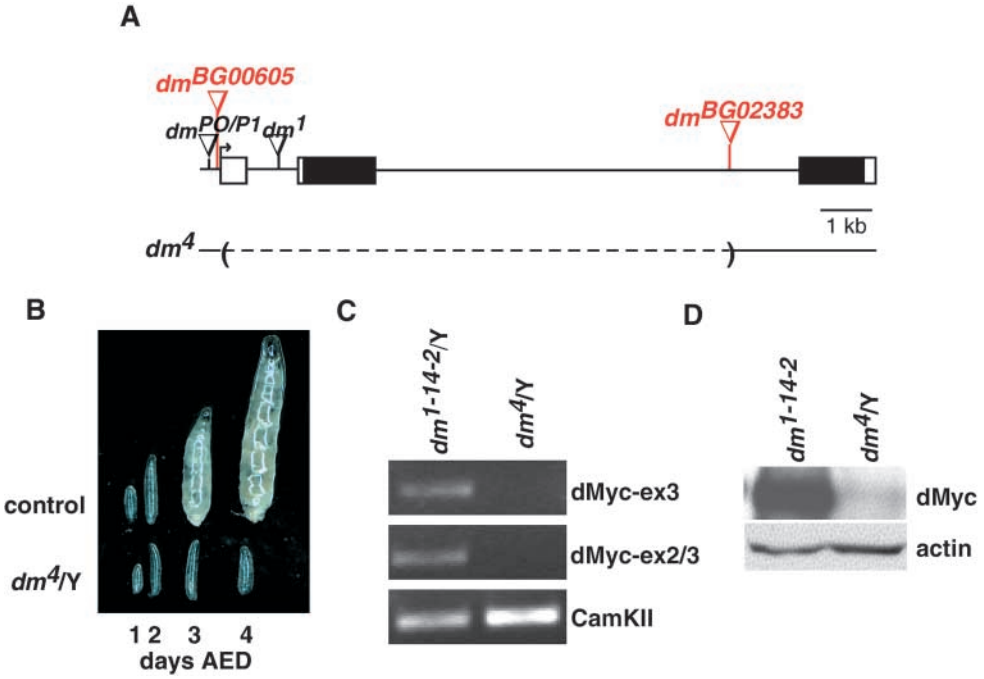


Fig. 1. Mutants with reduced dMyc have a larval growth defect. (A) *dm* gene. The dm^4 deletion, the positions of the P-elements used in the excision screen (in red), and the P-element and gypsy element that define the hypomorphic alleles $dm^{P1/P0}$ and dm^1, respectively, are indicated. Black boxes, coding region; arrow, the transcription start site. (B) Comparison of age-matched dm^4/Y hemizygous mutants with control dm^{1-14-2}/Y (see text) hemizygous males. (C) RT-PCR analysis of RNA from larvae of the indicated genotypes, harvested at 24 hours AED, using primers to detect regions across the exon 2/3 boundary and within exon 3 of dMyc. CaMKII is a loading control. (D) Western blot of extracts from larvae of the indicated genotypes, harvested 24 hours AED.

growth, we sought to isolate stronger dMyc alleles. By mobilizing two P-elements inserted in the *dm* gene on the X chromosome and carried in trans, we isolated dm^4 (Fig. 1A). The P-elements in P{w^{+mGT}=GT1}dm^{BG00605} and P{w^{+mGT}=GT1}dm^{BG02383} are inserted just upstream of the *dm* transcription start and near the 3′ end of the large second intron of the *dm* gene, respectively. PCR and sequencing analysis revealed that all of the sequence between the P-element insertion sites is deleted in dm^4 (Fig. 1A). Because this results in deletion of the transcription start site and the first two exons, we presume that dm^4 is a null mutant. Male mutant larvae, hemizygous for dm^4, hatch at approximately the same rate and size as control larvae, suggesting that their embryonic development is normal. As larvae, however, they have a strong growth defect and are noticeably smaller within 24 hours after hatching (48 hours after egg deposition-AED) (Fig. 1B). These larvae continue to develop and molt into the second instar, during which they stop growing. Most dm^4 mutant larvae die soon after arrest.

To determine whether any dMyc transcript is produced from the remaining third exon in dm^4, RT-PCR was performed using RNA from larvae harvested 24 hours AED. As a control we used larvae from dm^{1-14-2}, a phenotypically wild-type line isolated in a screen for excision of the P-element from P{w^{+mGT}=GT1}dm^{BG02383} alone (see Materials and methods). Using a primer pair that bridges the exon 2/exon 3 boundary, and therefore would not amplify a product from dm^4, which is missing exon 2, we determined that no maternal dMyc transcript remains in dm^4. In addition, no product was detected in dm^4 using a primer pair within the third exon. Given the absence of any remaining maternal transcript, which would also be detected by this primer pair, this indicates that dm^4 does not produce a truncated transcript containing exon 3 sequences and is therefore a null mutant. Consistent with the degradation of maternal *dm* transcripts by 24 hours AED, maternal dMyc protein is undetectable by western blotting in dm^4 mutant larvae harvested 24 hours AED (Fig. 1D). dm^4 mutant males can be rescued to adulthood by a transgene in which the tubulin promoter drives dMyc expression (data not shown), indicating that the dm^4 phenotype is due to the deletion within the *dm* gene. These results demonstrate the importance of dMyc for larval growth, which is primarily dependent on endoreplication.

dMyc and dMnt are expressed in larval endocycling tissues in complementary patterns

We next used antibody staining to investigate the expression patterns for dMyc and dMnt during larval stages. In several endoreplicating tissues, including the salivary gland (Fig. 2), the proventriculus and gastric cecae (data not shown), dMyc

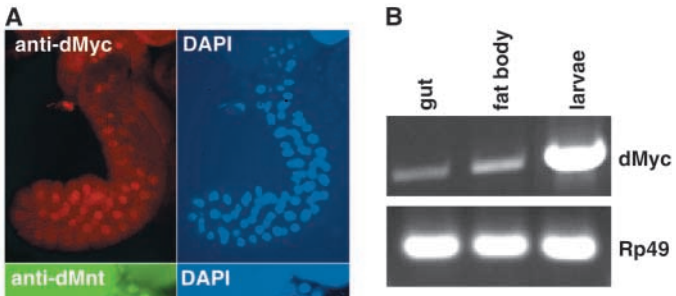
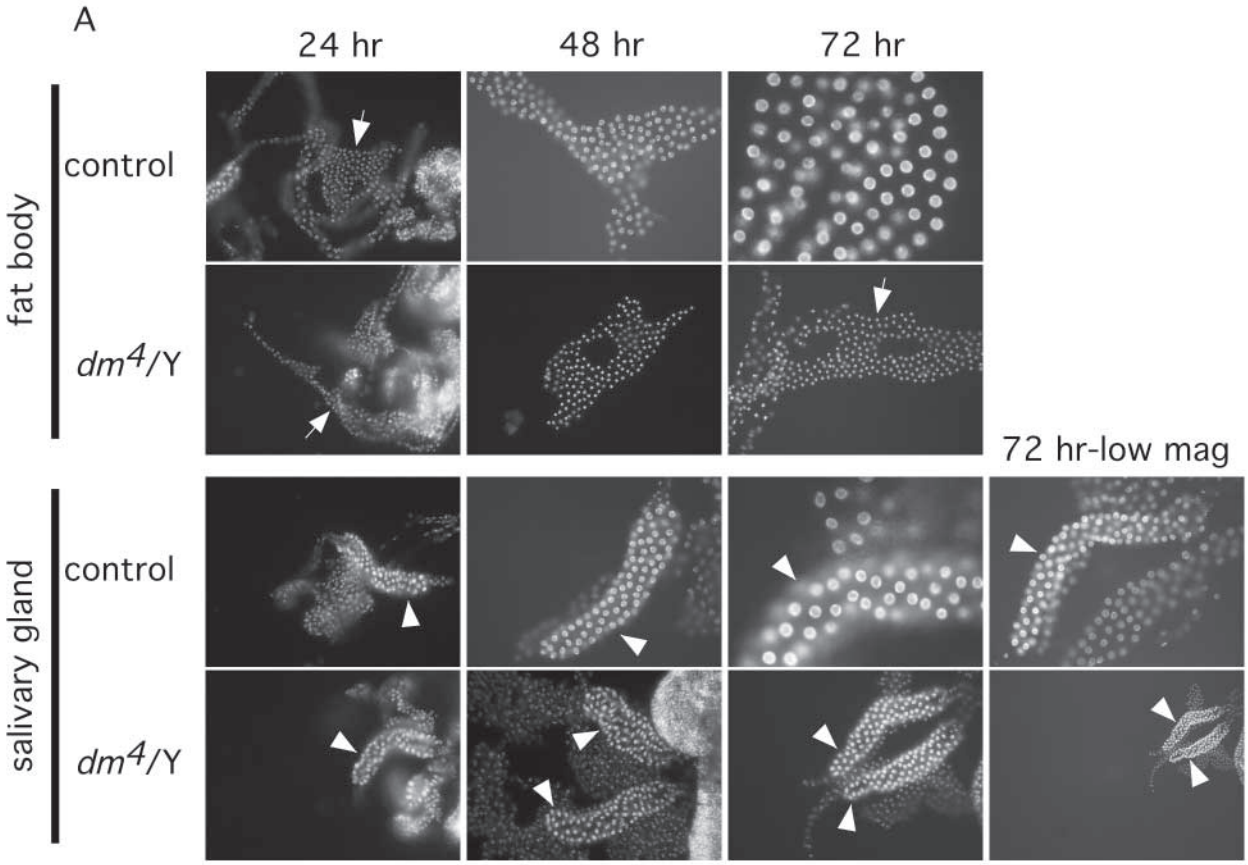


Fig. 2. dMyc and dMnt are expressed in endoreplicating tissues. (A) Salivary glands from wandering third instar larvae were stained with antibodies to dMyc or dMnt (left panels), and DAPI (right panels). dMyc is expressed at higher levels in distal cells while dMnt is expressed at higher levels in proximal cells. (B) RT-PCR to detect dMyc transcript in gut or fat body from whole wandering third instar *w; iso2,3* larvae. Rp49 is a loading control.



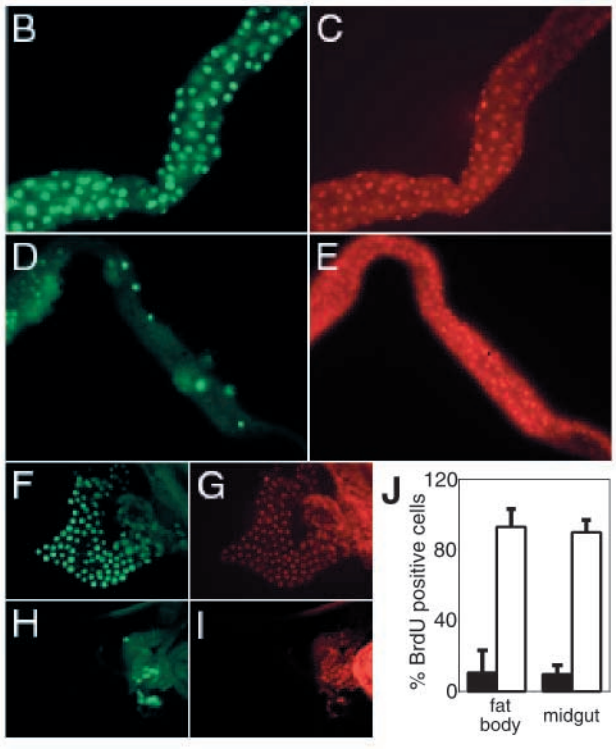


Fig. 3. *dm*^{4} mutants have reduced nuclear size. (A) DAPI stained fat body (arrows) and salivary glands (arrowheads) from *dm*^{4} /Y hemizygous mutants or control *dm*$^{1–14-2}$/Y hemizygous males, harvested at the indicated times AED. Lower magnification (72 hr-low mag) shows the difference in salivary gland size by 72 hours AED. (B-I) Control *dm*$^{1-24-2}$/Y (B,C,F,G) and *dm*^{4}/Y (D,E,H,I) larvae were fed BrdU from 30 to 48 hours AED. Midgut (B-E) and fat body (F-I) were stained with anti-BrdU (B,D,F,H) and DNA was visualized with propidium iodide (C,E,G,I). (J) The percentage of BrdU-positive cells in control (white bars) or *dm*^{4}/Y (black bars) fat body and midgut tissue was determined for the experiment above. Fifteen to 21 tissue samples were analyzed for each bar of the graph, each consisting of an average of ~50 cells.

and dMnt display inverse patterns of expression. dMyc expression persists in the distal region of the third instar salivary gland, whereas dMnt expression is limited to the proximal region (Fig. 2). In both cases, DAPI stained nuclei are visible throughout. The distal region of the salivary gland undergoes more endoreplication than the proximal region (Berendes and Ashburner, 1978), resulting in approximate final ploidies of 2048C and 1024C (Hammond and Laird, 1985). Because of this defined difference in local endoreplication, we chose to focus our studies on this tissue. BrdU labeling revealed that the cells that cycle last in the salivary gland are those at the distal region of the gland (data not shown), which both express dMyc longer and attain the highest final ploidy. The fact that sustained expression of dMyc correlates with this extra endoreplication suggests that dMyc may be linked to endoreplication. Larvae that were stained for both dMyc expression and BrdU incorporation showed that most cells that express dMyc are also labeled with BrdU and thus are actively endoreplicating (data not shown). Conversely, dMnt is expressed in proximal salivary gland cells that have exited the endocycle.

To determine whether dMyc is also expressed in other endoreplicating larval tissues, we performed RT-PCR on specific tissues dissected from wild type third instar larvae. This demonstrated that dMyc is also expressed in larval fat body and midgut (Fig. 2B), indicating that dMyc is expressed in many distinct types of endoreplicating cells. This is supported by the expression of dMyc in embryonic gut and salivary gland cells, which also endoreplicate (Gallant et al., 1996; Schreiber-Agus et al., 1997).

dMyc is required for DNA endoreplication

To determine whether dMyc is required for endoreplication, we compared nuclear size of endocycling cells in *dm*^{4} mutant and control animals. Larval tissues were fixed and stained with DAPI to visualize the DNA. At approximately the time of hatching (24 hours AED), the nuclei of *dm*^{4} mutant salivary gland, fat body, and gut tissue appeared similar to those of control tissue (Fig. 3A and data not shown). However, by 48 hours AED, nuclei of mutant tissue were noticeably smaller than control nuclei and the difference was even more pronounced at 72 hours AED (Fig. 3A). In addition, the overall size of the *dm*^{4} mutant salivary gland and gut was smaller than control (Fig. 3A), consistent with a reduction in cell size.

That *dm*^{4} mutant nuclei fail to attain normal size suggests that they fail to accumulate normal levels of DNA and may undergo significantly fewer endocycles than wild-type cells. To investigate this directly, we used BrdU to label S-phase nuclei. *dm*^{4} mutant and control larvae were fed BrdU from 30-48 hours AED and endoreplicating tissues were fixed and stained to reveal BrdU incorporation. In anterior midgut and fat body, most control nuclei were labeled with BrdU, indicating that they underwent at least one round of DNA replication during the labeling period (Fig. 3B,C,F,G). By contrast, very few *dm*^{4} mutant nuclei were labeled with BrdU (Fig. 3D,E,H,I). When we quantified the percentage of BrdU-positive cells for many tissue samples, we found that although ~90% of control midgut and fat body cells were labeled with BrdU, only about 10% of *dm*^{4} mutant midgut and fat body cells were BrdU positive (Fig. 3J). Thus, the failure of *dm*^{4} mutant nuclei to reach normal size and accumulate normal levels of DNA results from a dramatically reduced rate of endocycling.

Ectopic expression of dMyc and dMnt have opposing effects in endoreplication

Endogenous expression patterns suggest opposing roles for dMyc and dMnt in larval endoreplication (Fig. 2A). To test this hypothesis, we used two techniques to ectopically express dMyc or dMnt UAS-regulated transgenes in endoreplicating larval cells. The first employed the Gal4/UAS system (Brand and Perrimon, 1993) to express dMyc or dMnt in salivary gland and fat body, under the control of Ptc-Gal4 or Adh-Gal4, respectively. Second, we used the FLP/Gal4 system to induce expression of dMyc and dMnt in random clones of cells throughout the larva, which are identifiable by co-expression of nuclear-localized GFP (Neufeld et al., 1998; Pignoni and Zipursky, 1997). Although this system is regulated by the heat-shock promoter, heat shock-independent activation of Gal4 occurs in a small number of cells in various tissues, including fat body and gut, before the onset of larval growth and endoreplication (Britton et al., 2002). Because these heat shock-independent events occur early, they result in transgene

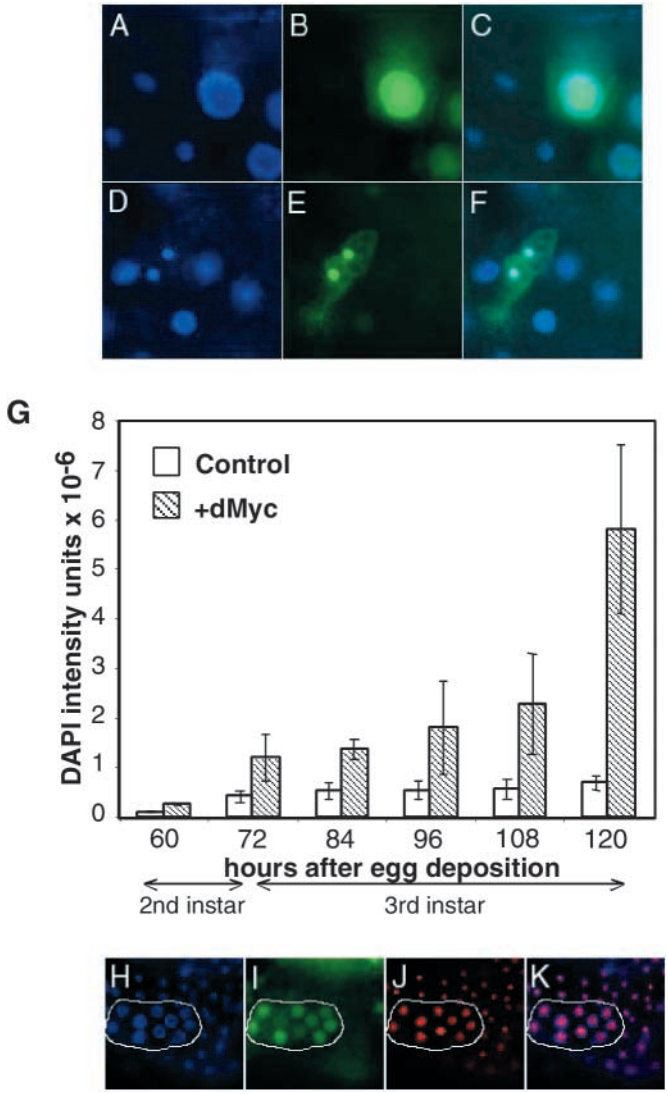


Fig. 4. dMyc promotes and dMnt blocks endoreplication. Flp/Gal4 was used to clonally express dMyc (A-C,G-K) or dMnt (D-F) with GFP (green). DNA is stained with DAPI (blue). (A-F) Heat shock-induced clones in fat body from third instar larvae; DNA and GFP images are merged in C and F. (G) Quantification of nuclear DAPI fluorescence. DAPI stained fat body nuclei from larvae of the indicated ages were analyzed by deconvolution microscopy and heat shock-independent GFP marked dMyc-expressing cells were compared with neighboring non-GFP control cells. (H-K) Fat body from a second instar larva containing a heat shock-independent dMyc expressing FLP/Gal4 clone [marked with GFP (I) and outlined in white] was stained with DAPI (H) and anti-fibrillarin (J). DAPI and anti-fibrillarin images are merged in K.

expression throughout larval development. Using both of these techniques, we found that ectopic dMyc and dMnt had opposite effects. dMyc drove ectopic endoreplication and resulted in larger cells with a higher ploidy than normal in fat body (Fig. 4A-C) and salivary gland (not shown) cells, whereas ectopic dMnt limited the number of endocycles, leading to smaller fat body (Fig. 4D-F) and salivary gland cells (not shown) with decreased final ploidy.

To more carefully study the effects of ectopic dMyc

expression, we quantified the increase in ploidy caused by dMyc overexpression. Deconvolution microscopy was used to measure the fluorescent intensity of DAPI stained nuclei, which is expected to reflect DNA content and thus the ploidy of the cell (Smith and Orr-Weaver, 1991). To verify this, we demonstrated that DAPI intensity is proportional to DNA concentration by comparing the ratio of spectrophotometrically determined concentrations of isolated DNA to nuclear DAPI fluorescence for salivary glands of different ploidy (data not shown). To determine the progression of the increase in ploidy induced by ectopic dMyc, we quantified DAPI fluorescence of fat body nuclei in heat shock-independent FLP/Gal4 clones, in which high levels of ectopic dMyc expression were driven by the actin promoter. We analyzed larvae harvested at 12 hour intervals, beginning in the second instar at 60 hours AED. The DAPI fluorescence of cells with ectopic dMyc was determined relative to neighboring cells in the same region of the fat body without ectopic dMyc (Fig. 4G). Ectopic dMyc expression led to a dramatic increase in final DNA content. Whereas fat body normally reaches a ploidy of 256C (Laird, 1980; Richards, 1980), we estimate that fat body cells overexpressing dMyc reached a ploidy of about 2048C, which represents three ectopic endocycles. To be sure that the increase in DNA content induced by ectopic dMyc expression occurred entirely during larval stages, we quantified DNA in cells from newly hatched larvae prior to feeding and thus endocycle entry. By comparing neighboring cells with and without ectopic dMyc, we determined that ectopic dMyc did not increase DNA content prior to the initiation of larval endocycles (data not shown). We conclude that dMyc overexpression increases the rate of endocycling, as reflected by the establishment of a higher ploidy in younger larvae. In addition, fat body and salivary gland cells expressing ectopic dMyc continue to actively endoreplicate later in development than control cells (Table 1 and Fig. 4G). For example, in third instar larvae, control fat body ploidy increases very modestly when compared with cells overexpressing dMyc (Fig. 4G).

To determine whether ectopic dMyc altered the normal pattern of endoreplication, we analyzed BrdU incorporation in salivary glands overexpressing dMyc driven by Pct-Gal4 and compared these with control glands. Although the salivary glands overexpressing dMyc continued to incorporate BrdU later in development than control salivary glands (Table 1), they maintained the characteristic pattern of BrdU incorporation, including the distal cells being the last to cycle at the end of endoreplication (data not shown). In summary, these studies support the model suggested by loss-of-function studies, endogenous expression patterns and studies in mitotic cells (Johnston et al., 1999) (L. Loo, J. Secombe and R.N.E., unpublished) in which dMyc drives the endocycle and cellular growth while dMnt serves to block the endocycle and growth.

dMyc influences nucleolar size

During our analysis of larval cells expressing ectopic dMyc, we observed what appeared to be abnormally large nucleoli. One of the hallmarks of vertebrate Myc overexpression in tumor cells is enlarged nucleoli (Abrams et al., 1982; Hann et al., 1983). To examine this, we stained cells with an antibody to fibrillarin (Aris and Blobel, 1988), which is involved in rRNA processing and ribosome assembly (Tollervey et al., 1993) and has been reported to be a transcriptional target of dMyc (Orian et al., 2003) and vertebrate Myc (Coller et al., 2000). Anti-fibrillarin staining of fat body overexpressing dMyc confirmed that nucleoli were abnormally large and expressed increased amounts of fibrillarin (Fig. 4H-K). This suggests that one mechanism by which dMyc drives growth and endoreplication is to increase the rate of ribosome biogenesis.

Table 1. Ectopic dMyc causes the period of endoreplication to extend later in development

	BrdU–	BrdU+
Salivary gland labeling*		
Control	71%	29%
dMyc	16%	84%
Fat body cell labeling†		
Control	98%	2%
dMyc	68%	32%

Late pre-wandering third instar larvae were labeled with BrdU for 5 hours prior to dissection.

*Gal4 regulated by the Ptc promoter was used to drive ectopic GFP (control) or dMyc and GFP (dMyc) in salivary glands.

†Gal4 regulated by the Adh promoter was used to drive ectopic GFP (control) or dMyc and GFP (dMyc) in fat body cells.

Cells overexpressing dMyc undergo a normal endocycle

The endocycle consists of discrete periods of DNA synthesis separated by gap phases. dMyc might increase the rate of endoreplication by eliminating the gap phase or by speeding up the G/S cycle. Both salivary gland and fat body overexpressing dMyc contain a subset of cells that fail to incorporate BrdU (Table 1 and Fig. 5A-D), although their nuclear size indicates previous endoreplication. From this we conclude that dMyc-overexpressing cells display discrete S-phases separated by a gap phase.

We also investigated whether dMyc overexpression would deregulate the oscillating expression of Cyclin E, an important promoter of S-phase initiation. To determine whether Cyclin E protein oscillates in control and dMyc-overexpressing larval tissues, we stained third instar larvae with anti-Cyclin E antibodies. We detected cells with both high and low levels of cycle E protein in wild-type and dMyc-expressing fat body (Fig. 5E) and salivary gland (data not shown), indicating that Cyclin E protein oscillates. These experiments indicate that endocycles driven by ectopic dMyc are in some respects normal, with Cyclin E oscillations that result in a cell cycle with alternating S- and gap phases.

dMyc growth effects are tightly linked to the cell cycle

Our studies of endoreplicating tissues, as well as our earlier work on mitotic imaginal disc cells (Johnston et al., 1999), have demonstrated that ectopically expressed dMyc drives both cellular growth and endocycle progression. To further study the mechanism by which this occurs, we asked whether ectopically expressed dMyc could overcome endoreplication arrest imposed by the expression of specific transgenes. We were particularly interested in whether induction of growth and endoreplication by ectopic dMyc could be uncoupled. Ptc-

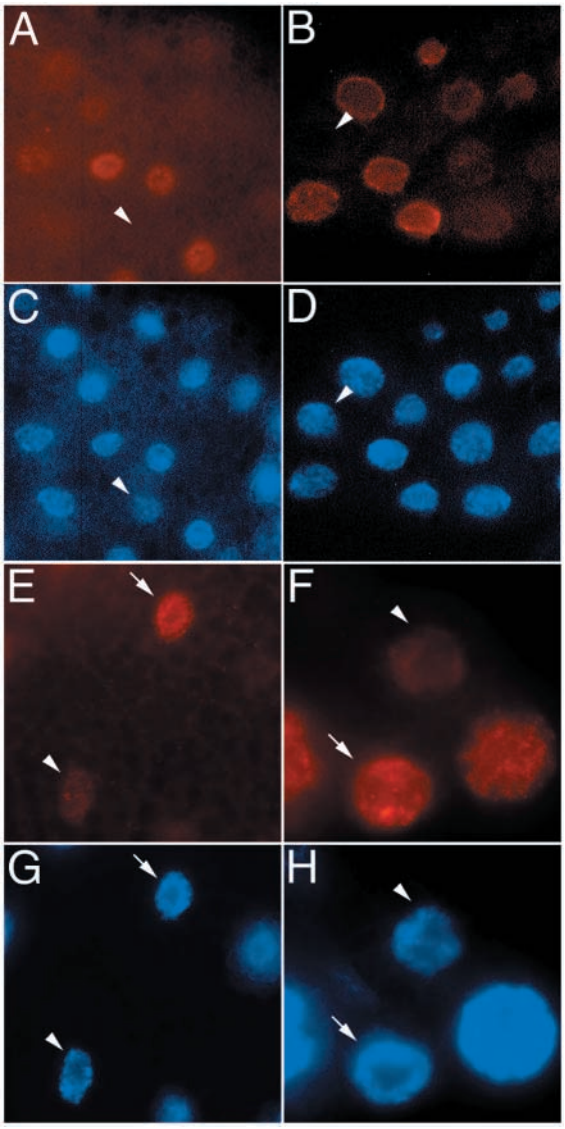


Fig. 5. DNA replication and Cyclin E protein levels oscillate in dMyc-expressing cells. (A-D) Control larvae (A,C) and larvae expressing dMyc driven by Adh-Gal4 (B,D) were fed BrdU for 5 hours and dissected at late second instar. Fat body was stained with anti-BrdU (A,B) and DNA was visualized with DAPI (C,D). Arrowheads indicate nuclei that did not label with BrdU. (E,F) Fat body from third instar control larvae (E,G) or larvae expressing dMyc driven by Adh-Gal4 (F,H) was stained with anti-Cyclin E (E,F) and DAPI (G,H). Cyclin E is expressed at high (arrows) and low (arrowheads) levels in control and dMyc-expressing cells.

Gal4, which is predominantly expressed in the salivary gland throughout larval development, was used to drive expression of different transgenes. The salivary glands of wandering third instar larvae were analyzed by staining with DAPI and anti-fibrillarin antibodies to visualize DNA and nucleoli, respectively. Continuous ectopic expression of Cyclin E blocks endoreplication and salivary gland growth (Follette et al., 1998; Weiss et al., 1998) (Fig. 6A,B), as does ectopic expression of vertebrate p21, a cyclin-dependent kinase inhibitor (Fig. 6A,C). It is thought that continuous high levels of active CyclinE/cdk2 complexes generated by Cyclin E overexpression block the 'resetting' of origins of replication, and thus S-phase initiation, in endoreplicating cells (Follette et al., 1998). Overexpression of p21 inhibits the activity of Cyclin E/cdk2 complexes, which are required to progress through the G/S transition. When dMyc was co-expressed with Cyclin E or p21, a slight increase in DNA and fibrillarin staining was observed (compare Fig. 6I,M with Fig. 6J,N and Fig. 6K,O), with little increase in the overall size of the salivary gland (compare Fig. 6F with 6B and Fig. 6G with 6C). Quantification of DAPI fluorescence by deconvolution microscopy indicated that co-expression of dMyc with Cyclin E or p21 increased the nuclear DNA content approximately twofold. By contrast, the nuclear DNA content of control salivary glands was ~25 times higher. These results suggest that ectopic dMyc is insufficient to drive significant endoreplication or cell growth when the cell cycle itself is arrested.

dMyc can bypass a block to endoreplication imposed by defective insulin signaling

The insulin signaling pathway has been shown to be an important growth mediator in *Drosophila* (reviewed by Johnston and Gallant, 2002; Oldham and Hafen, 2003). We tested whether ectopic dMyc expression could overcome the growth inhibitory effect imposed by reduced insulin signaling. Ectopic expression of p60, the PI3 Kinase adaptor protein, which inhibits insulin receptor signaling (Weinkove et al., 1999), blocked both cellular growth and endoreplication in the salivary gland (Fig. 6D,L). When co-expressed with p60, dMyc was able to partially rescue this block (Fig. 6H,P), increasing the nuclear DNA content by approximately sixfold, although less growth and endoreplication was induced than when dMyc was expressed in otherwise wild type cells (Fig. 6I,M). These results suggest that dMyc can induce some endoreplication and growth independently of the PI3 kinase branch of the insulin signaling pathway. By contrast, dMyc induced little endoreplication or growth in larvae that were mutant for either of the translation initiation factors eIf4a or eIf4e (data not shown). Our finding that ectopic dMyc either drives both endoreplication and cell growth, as when PI3 kinase activity is compromised, or induces neither, as when the endocycle is blocked by Cyclin E or a cdk inhibitor, suggests that dMyc-induced cellular growth and endoreplication are tightly coupled. This is consistent with the arrest of both growth and endoreplication in the dm^4 mutant.

Discussion

Previously isolated alleles of *dm* develop more slowly than do wild type and produce smaller than normal adults, indicating that dMyc function is important for proper growth of the animal (Gallant et al., 1996; Johnston et al., 1999; Schreiber-Agus et al., 1997). Because endoreplicating cells make up the bulk of larval tissues and are therefore predominantly responsible for the dramatic growth of the larva (see Gilbert, 2003), we chose to study potential roles of *Drosophila* Myc, as well as its putative antagonist dMnt, in larval growth and endoreplication. Our studies indicate that dMyc and dMnt are profoundly involved in the tightly coupled processes of DNA endoreplication and the accompanying increase in cell size.

Analysis of a new *dm* mutant allele

To determine whether there is a requirement for dMyc in driving endoreplication, we isolated dm^4, a null allele of *dm*. The failure of dm^4 mutants to grow beyond the second instar (Fig. 1B) indicates that dMyc is required for growth at the organismal level. We presume that maternally deposited *dm* gene products, or other maternal products, are sufficient for development of the embryo. The fact that maternal *dm* transcripts and protein are undetectable by the time of hatching suggests that dMyc is not required for the initiation of larval growth and may not be required for completion of embryogenesis, although it is possible that a small amount of residual maternal dMyc supports the growth of dm^4 mutant larvae prior to their arrest. The massive growth that takes place during larval development is tightly coupled to the endoreplication that takes place in all larval tissues except the imaginal discs and nervous system (Galloni and Edgar, 1999; Smith and Orr-Weaver, 1991). Our finding that dMyc and dMnt are expressed in distinct groups of cells in these tissues is suggestive of roles in promoting (dMyc) and limiting (dMnt) endoreplication (Fig. 2) and suggests that the failure of dm^4 mutants to grow is the result of loss of dMyc in endocycling tissues.

Larval growth involves both cytoplasmic growth and DNA endoreplication (Edgar and Orr-Weaver, 2001). The reduced rate of BrdU incorporation in early larval tissues and the failure of dm^4 mutant nuclei to grow suggest that these mutants undergo reduced DNA replication. The fact that mutant cells and larvae are smaller than age-matched controls indicates that that there is also a growth defect. Thus, directly or indirectly, dMyc is required for both growth and DNA replication during endoreplication (Fig. 3).

Overexpression of dMyc and dMnt have opposite effects on endoreplicating cells

Consistent with our finding that dMyc loss of function negatively affects endoreplication and growth, overexpression of dMyc drives both cellular growth and DNA replication (Fig. 4A-C,G). By contrast, overexpression of the *Drosophila* ortholog of Mad, dMnt (L. Loo, J. Secombe and R.N.E., unpublished), blocks cellular growth and DNA replication (Fig. 4D-F). These results are consistent with a model in which dMyc and dMnt act antagonistically, with dMnt binding and repressing the genes required for endoreplication that dMyc activates. We find that dMnt is normally highly expressed in the third instar salivary gland and other tissues that have exited the endocycle, indicating that dMnt-mediated gene repression may be necessary for this transition. However, dMnt, the only Mad family ortholog in *Drosophila*, is non-essential. dMnt null mutants develop normally into adults with modestly increased body weights and shorter lifespans (L. Loo, J. Secombe and R.N.E., unpublished). Although the increased body weight of dMnt mutants is consistent with negative regulation of growth by dMnt, we have not observed altered endoreplication in mutants (L. Loo, J. Secombe, unpublished), suggesting that negative regulators of endoreplication other than dMnt must exist.

dMyc influences growth and S phase entry in endoreplicating cells

A role for dMyc in regulating both organismal size and the size of mitotically dividing cells has been previously reported (Johnston et al., 1999). We show that in endoreplicating cells ectopic expression of dMyc results in increased cytoplasmic and nuclear volume, as well as in enlarged nucleoli, as detected by increased anti-fibrillarin staining (Fig. 4H-K). Fibrillarin has been implicated as a Myc target gene in both vertebrate and *Drosophila* cells (Coller et al., 2000; Orian et al., 2003) and its augmented expression is consistent with the notion that dMyc/Myc promotes ribosome biogenesis (Tollervey et al., 1993). The *pitchoune* gene, which encodes a putative RNA-helicase localized to the nucleolus, is also induced by ectopic dMyc, and *pit* null mutants have a severe larval growth defect similar to dm^4 mutants (Zaffran

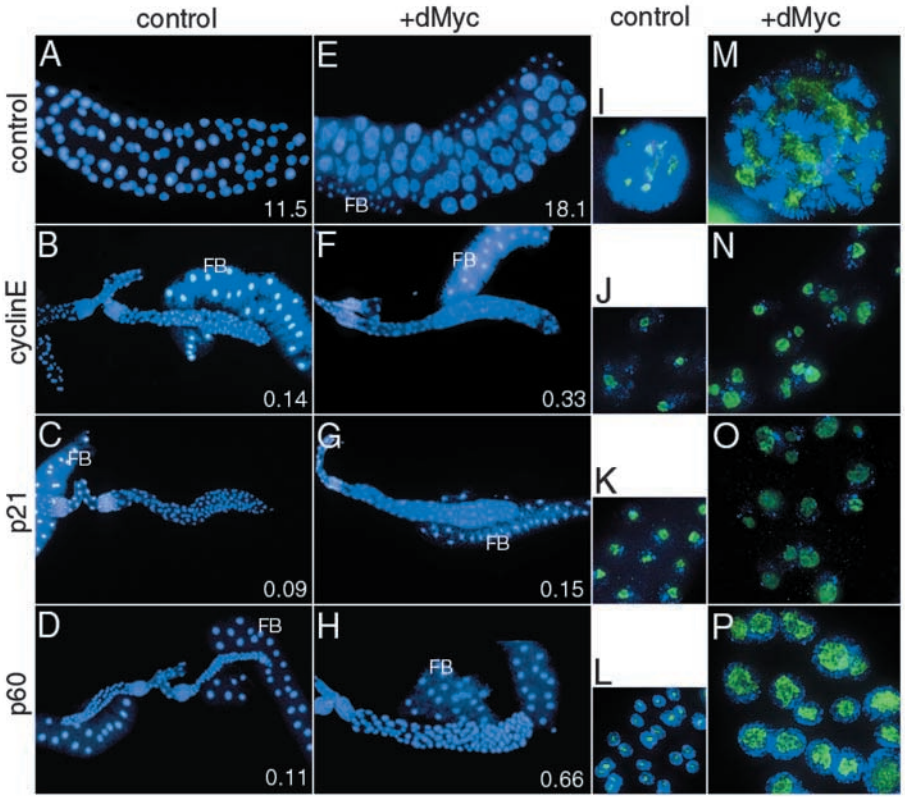


Fig. 6. dMyc induced growth and endoreplication is blocked by Cyclin E and cdk overexpression but only partially by loss of PI3K activity. (A-P) DAPI stained salivary glands with attached fat body (FB) from wandering third instar larvae with or without (control) the indicated transgenes driven by ptc-Gal4. (I-P) Nuclei double stained with DAPI (blue) and anti-fibrillarin (green) are shown at higher magnification. Numbers in the lower right corner (A-H) indicate the ratio of median DAPI fluorescent intensity of posterior salivary gland nuclei to a single fat body nucleus.

et al., 1998). The mammalian ortholog of *pit*, MrDB (DDX18), has been identified as a direct target of c-Myc (Grandori et al., 1996). In addition, many other known and suspected targets of the Myc family are involved in this process (Boon et al., 2001; Guo et al., 2000; Neiman et al., 2001; Orian et al., 2003).

S-phase of the endocycle is initiated by the activity of Cyclin E/cdk2 (Follette et al., 1998) but endoreplication can be blocked by continuous ectopic expression of Cyclin E or the human cdk inhibitor p21 (Follette et al., 1998; Weiss et al., 1998) (Fig. 6). It is thought that Cyclin E levels must drop after each S-phase and then increase again prior to the next S-phase to allow reinitiation of DNA replication. In mitotic cells, this prevents more than one round of DNA replication from occurring during each cell cycle. In endoreplicating cells it results in discrete S-phases separated by a gap phase. Ectopic p21 is likely to inhibit the activity of cdk2 even in the presence of Cyclin E (de Nooij et al., 1996). The extra endocycles driven by ectopic dMyc appear to be normal, in that there are discrete periods of DNA replication and Cyclin E appears to oscillate (Fig. 5E-H). As ectopic dMyc induces cells to accumulate high levels of DNA earlier in development (Fig. 4G), we presume that S-phases and Cyclin E oscillations occur more frequently than normal. It is also possible that the S-phases are shorter and that Cyclin E peaks at higher levels when ectopic dMyc is present. When co-expressed with ectopic unregulated Cyclin E or p21, dMyc drives very little endoreplication (Fig. 6), suggesting that the cell cycle control exerted by oscillating Cyclin E/cdk2 activity is downstream of dMyc function. Consistent with this, ectopic dMyc can post-transcriptionally increase Cyclin E levels in wing discs (Prober and Edgar, 2000) and studies in mammalian cells suggest that Myc can indirectly induce Cyclin E expression (Steiner et al., 1995). Microarray analysis did not identify Cyclin E as a transcriptional target of dMyc (Orian et al., 2003), suggesting that the transcriptional oscillation of Cyclin E is not directly regulated by dMyc. Thus, dMyc is unable to drive endoreplication in the absence of normal cdk activity. Although the level of fibrillarin staining was not quantified, dMyc appears to drive somewhat more nucleolar growth than DNA accumulation when co-expressed with Cyclin E or p21 (Fig. 6B-O), indicating that dMyc may be able to drive a limited amount of nucleolar growth in the absence of DNA replication.

The *Drosophila* insulin signaling pathway is also essential for growth. Mutations in the receptor InR and downstream components of the pathway, including Dp110, a PI3 kinase homolog, cause larval growth defects (Chen et al., 1996; Weinkove et al., 1999). We found that when PI3 kinase signaling was blocked by ectopic expression of p60, dMyc was still able to induce a significant amount of cellular growth and DNA replication (Fig. 6). This suggests either that dMyc is downstream of PI3 kinase signaling or that dMyc and dDp110 represent independent pathways that are both essential for growth. Recent studies have found that Dp110 or InR overexpression did not result in increased dMyc transcription (B.E. and L. Li, unpublished) and that activated Ras increased dMyc levels and PI3 kinase activity via independent effector pathways (Prober and Edgar, 2002), suggesting that dMyc transcription is not downstream of the insulin signaling pathway. In addition, although ectopic expression of either dMyc or Dp110 leads to increased cell growth, the increase in nuclear size is more pronounced in response to dMyc whereas the increase in cytoplasmic volume is more pronounced in response to Dp110 (Saucedo and Edgar, 2002), further supporting the idea that dMyc and Dp110 regulate growth and endoreplication independently.

Defining dMyc pathways for growth and replication

dMyc overexpression augments cell growth in mitotic wing disc cells by shortening the mass doubling time. Such cells display a decrease in the length of G1 and a compensatory increase in the length of G2/M, resulting in a division time equal to that of control cells (Johnston et al., 1999). They retain their normal ploidy and show little effect on the length of S phase. We show here that in endoreplicating cells, dMyc drives both cellular growth and DNA replication. What is the relationship of dMyc function to these processes? dMyc transcriptionally activates a wide range of genes involved in ribosome biogenesis, translation and metabolism, suggesting that the relationship of dMyc to growth is likely to be very direct. The absence of an effect of dMyc on S phase length and cell division rate in mitotic cells argues that perhaps the only role of dMyc is to regulate cell growth. Interestingly, the division rate of dMyc-overexpressing mitotic cells is increased by introduction of String, which accelerates G2/M, resulting in the generation of a larger number of cells. Perhaps in endoreplicating cells, which lack G2/M entirely, dMyc simply increases the growth rate thereby shortening the G1-S transition and leading to a higher rate of S phase entry. Because such cells are incapable of division, the net effect observed is larger cells with increased ploidy. In this model, dMyc is thought to augment endoreplication indirectly, through its promotion of growth. While this paper was in preparation Maines et al. (Maines et al., 2004) reported that dMyc is required during oogenesis for somatic and germ cell growth and endoreplication, but not for proliferation prior to the onset of endoreplication. The finding that dMyc mutant follicle cells exhibit reduced growth prior to endoreplication suggests that the defect in endoreplication may be secondary to the defect in cellular growth.

Alternatively, dMyc might affect endoreplication more directly. Although both mammalian and *Drosophila* Myc target genes are predominantly growth related, a smaller number of gene targets are involved in cell cycle control and DNA replication (Fernandez et al., 2003; Orian et al., 2003; Staller et al., 2001). Importantly, dMyc does not increase transcript levels of Cyclin E or the *Drosophila* E2F1 transcription factor, the only known limiting factors for endocycles in endoreplicating tissues (Orian et al., 2003). Our finding that the effect of dMyc on growth is attenuated when the cell cycle is blocked by continuous expression of Cyclin E or p21 (Fig. 6) indicates that dMyc-induced growth is tightly coupled to DNA replication, at least in endoreplicating cells. The large number and diversity of the genes identified as likely targets of Myc genes indicates that Myc activity impinges on a broad range of cellular functions that must be highly coordinated for proper cell behavior. Interestingly Myc overexpression has been reported to lead to endoreplication and polyploidy in human kertinocytes (Gandarillas et al., 2000). Furthermore, in murine fibroblasts treated with colcemid, Myc overexpression leads to abrogation of the G2/M checkpoint and marked polyploidy (Li and Dang, 1999). These results suggest that Myc function is

involved in controlling S-phase entry and G2/M in diverse vertebrate cell types. The *Drosophila* endoreplicating cell system should provide a good model for better defining the precise role of Myc in coordinating growth and cell cycle.

This work was supported by NIH grants GM51186 to B.A.E. and CA57138 to R.N.E., a Howard Hughes Graduate Fellowship to J.S.B., and a Viral Oncology Training Grant (PHS #CA09229) to C.Y. We thank Julie Secombe, Amir Oryan and members of the Eisenman and Edgar laboratories for discussion and critical reading of the manuscript. We are grateful to Leni Sue Carlos, John Little, Adrian Quintanilla, David McDonald and Margaret Cheng for technical assistance; Terry Orr-Weaver and John Aris for antibodies; and Peter Gallant, the Bloomington Stock Center and Berkeley Drosophila Genome Project for fly stocks.

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